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(54) Title: TRANSGENES WITH FLOURY2 GENE SIGNAL PEPTIDE AND TRANSGENIC PLANTS CONTAINING THE TRANSGENES

(57) Abstract

A transgene is disclosed that contains a polynucleotide segment coding for a portion of a signal sequence from a *fluory2* gene of maize linked to an agronomically high-value protein. Cereal plants that contain such a transgene also are disclosed, as are millet or sorghum plants that contain a maize *fluory2* gene.

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TRANSGENES WITH FLOURY2 GENE SIGNAL PEPTIDE AND TRANSGENIC PLANTS CONTAINING THE TRANSGENES

BACKGROUND OF THE INVENTION

The present invention relates to a transgene that contains a polynucleotide segment encoding at least a portion of a signal sequence from a *floury2* gene. For example, a transgene within the invention also can contain a second segment coding for an agronomically high-value protein, such that the transgene expresses a fusion protein comprised of the signal-sequence moiety and amino acid sequence of the high-value protein. The present invention also relates to cereal plants that contain a transgene, such as millet or sorghum plants containing a maize or other heterologous *floury2* gene.

Between 50% and 60% of the protein in maize kernels consists of a mixture of prolamin storage proteins known as "zeins," which are essentially devoid of lysine. This makes the seed nutritionally inferior for monogastric animals. The lysine deficiency of maize spurred extensive efforts to identify mutants with higher levels of this essential amino acid. The maize *floury2* (*fl2*) mutant was first described by Emerson *et al.*, and was reported to result from a semidominant mutation that causes a soft, starchy endosperm. CORNELL UNIVERSITY AGRICULTURAL EXPERIMENTAL STATION REPORT 180 (1935). (The contents of this document and all others mentioned herein are incorporated by reference.) The *fl2* allele occurs on the short arm of chromosome 4, and for many years it served as a useful genetic marker for the short arm of chromosome 4.

In 1964, *fl2* and another mutation in maize, *opaque2* (*o2*), took on special interest when it was reported that both of these mutations lead to a substantial increase in lysine content of maize seeds. Nelson *et al.*, *Science*

150: 1468-70 (1965), and Mertz *et al.*, *Science* **145**: 279-80 (1964). Kernels in normal maize genotypes average around 0.20 to 0.25% lysine, while kernels from *o2* and *fl2* mutations have lysine contents of 0.3 to 0.35%. But the soft starchy endosperm associated with the *fl2* and *o2* phenotypes causes the kernels to be susceptible to mechanical damage, which creates a higher susceptibility to insect and fungal damage. Consequently, neither mutant gained widespread commercial application.

For many years, *o2* and *fl2* were considered to be defects of genes regulating zein synthesis. This conclusion was based on the significant and fairly specific effect these mutations have on storage protein synthesis. Both *o2* and *fl2* reduce zein synthesis by about 50% of the wild type level, with the *o2* mutation specifically affecting the 22-kDa α-zeins, and the *fl2* mutation equally affecting synthesis of all classes of zeins.

Other distinctive biochemical differences have been reported for *o2* and *fl2* mutants. Protein bodies in both *o2* and *fl2* are smaller than normal, but *fl2*-encoded protein bodies are asymmetrical and misshapen compared to the spherical protein bodies of normal and *o2* endosperm. Lending & Larkins, *Plant Cell* **1**: 123-133 (1989). The *o2* mutation is recessive, while the *fl2* mutation is semidominant, with the severity of the phenotype correlated to dosage of the mutant allele.

The hypothesis that *o2* is a zein regulatory gene was confirmed eventually, following its tagging with a transposable element. This led to the molecular cloning of *O2* and the demonstration that it encodes a leucine zipper-type transcription factor that binds the promoters of certain α-zein genes and controls expression of the 22-kDa family of α-zein genes. Schmidt *et al.*, *Science* **238**: 960-63 (1987). Numerous attempts to tag *fl2* by a similar strategy were unsuccessful, and the basis of the *fl2* defect remained unknown.

The failure to discern the nature of the *fl2* defect continued despite fairly extensive study of this mutant. Several studies noted the appearance of an unusual α-zein protein in *fl2* with a molecular weight of 24 kDa, higher than normal zein. Lee *et al.*, *Biochem. Genet.* **14**: 641-50 (1976); Soave *et al.*, *Maydica* **23**: 145-52 (1978); Galante *et al.*, *Mol. Gen. Genet.* **192**: 316-21 (1983). The level of this protein was found to be dependent on the dosage of the *fl2* allele. A high concentration of b-70, the maize homologue of the BiP chaperonin, also was reported to be associated with protein bodies in *fl2*. Zhang & Boston, *Protoplasma* **171**: 142-52 (1993). BiP is a member of the hsp-70 protein family that binds malfolded polypeptide chains. The level of b-70 is affected by the dosage of *fl2* double mutants, as is the degree to which the protein bodies become misshapen. It also was reported that in *o2/fl2* double mutants, the unusual 24-kDa α-zein was not synthesized and the morphology of the protein bodies was similar to that in *o2*. Thus, the *o2* gene was reported to be epistatic to *fl2*. Lopes *et al.*, *Mol. Gen. Genet.* **245**: 537-47 (1994).

Lopes *et al.* reported three α-zeins proteins in addition to the abnormal 24-kDa protein in the storage protein fraction of *fl2*, with molecular weights ranging from about 25 to 27 kDa. They also detected a restriction fragment length polymorphism (RFLP) linked to the *fl2* locus with a 22-kDa α-zein probe. They hypothesized that the characteristics of *fl2* might be a response to the accumulation of the defective 24-kDa protein, but were unable to prove that the accumulation of this protein was responsible for the *fl2* phenotype.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a fusion protein of a 21 amino acid signal sequence from *fl2* with a desired protein.

It is a further object of the invention to provide plants that contain an exogenous DNA sequence comprising this fusion protein, in which expression of the desired protein is increased in seeds of the plant.

It also is an object of the present invention to provide a method of increasing the content of essential amino acids in an animal feed without supplementation.

In accomplishing these and other objectives, there has been provided, in accordance with one aspect of the present invention, a cereal plant that contains a transgene comprised of (i) a first polynucleotide segment comprising a nucleotide sequence that encodes the amino acid sequence MATKILALLALLALLVSATNV and (ii) a second polynucleotide segment coding for a protein. In one preferred embodiment, polynucleotide segment (ii) has a high content of an amino acid selected from the group consisting of methionine, lysine, tryptophan and threonine, such that the amount of said amino acid in seeds of said cereal plant is increased as compared to seeds from otherwise identical plants that are not transformed. In another preferred embodiment, the first and second polynucleotide segments are operably linked to a promoter, such as the *f12* promoter, so that said cereal plant expresses both segments under the control of said promoter. Pursuant to other embodiments, the cereal plant can be a maize plant, where segment (ii) is not native to maize, or can be rice, wheat, barley, millet or sorghum, for example.

In accordance with other aspects of the present invention, there also is provided seed produced by a plant as described above and a feed product comprising meal obtained from such seed.

Pursuant to another aspect of the present invention, a transgene is provided that comprises (i) a first polynucleotide segment comprising the nucleotide sequence coding for the amino acid sequence MATKILALLALLALLVSATNV and (ii) a second polynucleotide segment coding for a protein. In a preferred embodiment, the transgene

additionally comprises a sequence of *f12* selected from one or both of nucleotides 761-3824 and 4613-8335 of Figure 1, described in greater detail below. In addition, a transgene is provided that comprises the *f12* promoter operably linked to a polynucleotide segment.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1A-1H together depict the nucleotide sequence of a clone of *f12*. Positions 1-760 and 8,336-10,539 are vector sequences, and positions 761-8,335 are the complete nucleotide sequence of *f12*.

Figure 2 shows the nucleotide sequence and deduced amino acid sequence of the coding region of *f12*, including the signal sequence. Numbers on the left correspond to position of the first amino acid of each line beginning with -21 to reflect the signal peptide, so that the -1 position is occupied by the C-terminal residue of the signal peptide and the +1 position is occupied by the first amino acid of the predicted mature polypeptide.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

It has been discovered that a gene encoding a 22-kDa α-zein protein, and not a regulatory gene, is responsible for the *f12* mutant phenotype discussed above. In

particular, it has been found that the 24-kDa protein identified in *fl2* mutants comprises the amino acid sequence of a 22-kDa α-zein plus an uncleaved, 21-amino acid signal peptide.

The complete nucleotide sequence of the *fl2* is shown in Figure 1, while Figure 2 depicts the nucleotide sequence and deduced amino acid sequence of the coding region of the 24-kDa protein. The deduced amino acid sequence of the signal peptide begins at position -21 in Figure 2, so that the -1 position is occupied by the C-terminal residue of the signal peptide and the +1 position is occupied by the first amino acid of the predicted mature polypeptide. The sequence of the signal peptide is MATKILALLALLALLVSATNV. A comparison of this deduced N-terminal amino acid sequence of the 24-kDa α-zein protein with other α-zeins has revealed an alanine to valine substitution at the C-terminal position of the signal peptide, a histidine insertion within the seventh α-helical repeat, and an alanine to threonine substitution with the same α-helical repeat of the protein. When an alanine codon is substituted for the valine codon of the mutant α-zein gene, the *in vivo* translated protein product is processed correctly in the presence of maize microsomes.

The signal peptide targets the α-zein protein to the lumen of the rough endoplasmic reticulum (RER). The signal peptide is retained on the 24-kDa α-zein precursor; that is, the 24-kDa α-zein is not processed in *fl2* endosperm. The 24-kDa α-zein is believed to remain anchored to the RER membrane, disrupting the normal biogenesis of protein bodies. In normal protein body development, zein proteins are retained within the ER where they coalesce into spherical bodies in which α-zeins are localized to the interior of a shell of cross-linked β- and γ-zein. Attachment of an α-zein to the RER membrane inhibits its movement into the interior of the protein body. More particularly, interaction of the RER-attached α-zein with the shell of β- and γ-zeins

disrupts the spatial organization of developing protein bodies by forming multiple foci for α-zein aggregation near the surface of the ER membrane, which the irregular budding that occurs in *fl2* protein bodies. The retention of the signal peptide on the 22-kDa α-zein also provides an explanation for the overexpression of BiP in *fl2*, since this would affect the normal folding of the protein.

The conversion of valine to alanine in the signal peptide of this α-zein provides an explanation for its retention on the protein, and for many of the phenotypic effects of the *fl2* mutation. According to von Heijne's "-3- rule" for signal peptides, the -1 position is critical for recognition by signal peptidase and is generally occupied by an uncharged amino acid with a small side chain. See von Heijne, *Eur. J. Biochem.* **133**: 17-27 (1983).

A point mutation in the signal peptide is consistent with the fact that only one *fl2* allele has been identified. A point mutation also helps to explain the difficulty in conventional approaches to tag *fl2* by transposon mutagenesis.

The complete *fl2* sequence contains 7575 base pairs, nucleotides 761-8,335 of Figure 1. The *fl2* coding region (open reading frame), including the stop codon, comprises nucleotides 3,825-4,613. This sequence has been transformed into maize. Transgenic seed that contained the gene expressed the *fl2*, 24-kDa zein, and seed segregating which did not have the *fl2* protein did not have the gene.

Since the coding region matches the sequence of *fl2*, 24-kDa protein, it is understood that the sequence shown in Figure 1 includes the promoter for *fl2*. Nucleotides between nucleotides 761 and about 3,824 in the sequence of Figure 1 encode the *fl2* promoter. Several motifs common to 22-kDa zein promoters are found in this region of the *fl2* sequence of Figure 1. For example, located upstream of the start of initiation is a sequence

5'-GTCATTCCAC-3'. The first nucleotide is at -300 with respect to the start of initiation. This corresponds to part of the sequence recognized by the *O2* gene product, also located 300 bp upstream of the start of initiation, as disclosed in Figure 5 of Schmidt *et al.*, *Plant Cell* **4**:689 (1992).

Similarly, Morton *et al.* refer to a prolamin-specific 5'-TGTAAAG-3' motif common to all zein genes of maize, commonly referred to as the "-300 box" by virtue of its location 300 bp from the start of translation. "Regulation of Seed Storage Protein Gene Expression," *in* SEED DEVELOPMENT AND GERMINATION (Kigel and Galili, eds.), New York: Marcel Dekker, Inc. (1995). A corresponding sequence is found at nucleotide 3500 in the *f12* sequence of Figure 1. Morton *et al.* also disclose a 5'-CATGCATG-3' element common to many seed-specific genes. This sequence is similar to the sequence 5'-CATGCGTG-3' of *f12*, which begins at nucleotide 3517 in Figure 1.

The retention of the 24-kDa protein on the RER and its accumulation in the endosperm leads to a concomitant decrease in the levels of other zein proteins and, hence, to a decreased level of total storage proteins. The reduction in total storage protein leads to the soft, starchy phenotype of *f12* and the reduction of the zein fraction as a percentage of total storage protein leads to an apparent increase in lysine content, since the other storage proteins in maize, such as globulins and albumins, have higher lysine contents.

The discovery of the nature of the *f12* defect provides the basis for seed progeny genetically engineered to express various phenotypes of agronomic interest. That is, a fusion protein of the 21 amino acid signal sequence from *f12* with a desired polypeptide can be used to provide increased expression of the desired polypeptide in seeds of a host plant. The region from 3,888-4,613 is replaced with the coding region (open reading frame) of the desired polypeptide.

Alternatively, *f12* sequences upstream and downstream of the coding region of *f12* can be used to drive expression of a desired polypeptide. In this case, the region from 3,825-4,613 in *f12* is replaced by the coding region of the desired polypeptide.

A wide variety of polypeptides can be fused to the *f12* signal sequence and/or to the regions upstream or downstream of the *f12* coding region, in order to achieve higher levels of expression and/or accumulation of the polypeptide than might otherwise be attained. The polypeptides may or may not be native to the plant in which they are being expressed. Illustrative of such polypeptides are short polypeptides of less than about 40 amino acids, polypeptides which are either unusually hydrophilic or hydrophobic or amphipathic, polypeptides which have unique solubility characteristics, polypeptides which have a unique three-dimensional structure, polypeptides which have motifs which include binding domains, polypeptides which have either very few or many disulfide bonds, polypeptides which have a high content of charged amino acids, polypeptides which have a particularly high content of specific amino acids, *e.g.* methionine, lysine, tryptophan or threonine, and polypeptides which have been altered from their native structure such that they might otherwise not be stable. DNA coding for the polypeptide may be modified to reflect preferred codon usage in the particular crop that is the target of the transformation.

A fusion protein of the signal peptide with a protein other than a zein protein should not affect the total amount of zein storage proteins that are accumulated by the plant. Inasmuch as it is the decrease in storage proteins in *f12* that is reported to give rise to the starchy endosperm characteristic of the *f12* mutant, a phenotype like that of the *f12* mutant is not expected when plants are transformed with a fusion protein of the *f12* signal peptide and a protein other than a zein protein.

Alternatively, the entire *f12* gene may be transformed into a plant that produces seeds that are mechanically stronger than maize. For example, sorghum and millet produce smaller and rounder seeds which are less affected by shear forces and, hence, to by any kernel weakening associated with *f12* expression. Expression of *f12* in these crops can provide seeds with improved digestibility without unacceptable levels of kernel weakening. It is expected that rice, wheat and barley likewise could accommodate *f12* expression in accordance with the present invention.

In accordance with the present invention, a DNA molecule comprising a transformation/expression vector is engineered to include the 21 amino acid signal sequence from *f12* and/or regions upstream or downstream of the *f12* coding region, and either the remainder of the *f12* gene or a DNA segment encoding a high-value protein as described above. A copy of the sequence of *f12* or of the 21 amino acid signal sequence from *f12* coupled to a desired high-value protein is placed into an expression vector by standard methods. The selection of an appropriate expression vector will depend upon the method of introducing the expression vector into host cells.

A typical expression vector contains: prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance gene to provide for the growth and selection of the expression vector in the bacterial host; a cloning site for insertion of the exogenous DNA sequence; eukaryotic DNA elements that control initiation of transcription of the exogenous DNA sequence, such as a promoter and an optional enhancer; and DNA elements that control the processing of transcripts, such as a transcription termination-polyadenylation sequence. The vector also could contain additional sequences that are necessary to allow for the eventual integration of the vector into a chromosome. For a general description of plant expression vectors, see Gruber *et al.*, "Vectors for

Plant Transformation," *in* METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY 89-119 (CRC Press, 1993).

Expression of the gene sequence is under the control of a promoter. Examples of suitable promoters are the promotor for the small subunit of ribulose-1,5-bis-phosphate carboxylase, promoters from tumor-inducing plasmids of *Agrobacterium tumefaciens*, such as the nopaline synthase and octopine synthase promoters, and viral promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters or the figwort mosaic virus 35S promoter. The promoter can be constitutive or inducible.

Especially preferred is a "seed tissue-preferred" or "seed tissue-specific" promoters, that is, promoters that drive high expression of the heterologous DNA segment in seed tissue where control of genes that are involved in seed metabolism is desired, and little or no expression in other parts of the plant. Manufacture of the protein encoded by the heterologous DNA segment in other parts of the plant needlessly expends the plant's energy. Examples of known seed tissue-preferred or seed tissue-specific promoters include the seed-directed promoters from the zein genes of maize endosperm. Pedersen *et al.*, *Cell* **29**: 1015 (1982). The *fl2* promoter is particularly preferred.

In addition to a suitable promoter, one or more enhancers are useful in the invention to increase transcription of the introduced DNA segment. The enhancer or enhancer-like element can be inserted into the promoter to provide higher levels of transcription. Examples of such enhancers include, *inter alia*, viral enhancers like those within the 35S promoter, as shown by Odell *et al.*, *Plant Mol. Biol.* **10**: 263-72 (1988), and an enhancer from an opine gene as described by Fromm *et al.*, *Plant Cell* **1**: 977 (1989).

Selectable marker genes, in physical proximity to the introduced DNA segment, are used to allow transformed cells to be recovered by either positive genetic

selection or screening. The selectable marker genes also allow for maintaining selection pressure on a transgenic plant population, to ensure that the introduced DNA segment, and its controlling promoters and enhancers, are retained by the transgenic plant.

Many of the commonly used positive selectable marker genes for plant transformation have been isolated from bacteria and code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or a herbicide. Other positive selection marker genes encode an altered target which is insensitive to the inhibitor.

A preferred selection marker gene for plant transformation is the BAR or PAT gene, which is used with the selecting agent bialaphos. Spencer *et al.*, THEOR. APPL. GENET., Berlin: Springer International, vol. 79, pp 625-631, 1990. Another useful selection marker gene is the neomycin phosphotransferase II (*nptII*) gene, isolated from Tn*5*, which confers resistance to kanamycin when placed under the control of plant regulatory signals. Fraley *et al.*, *Proc. Nat'l Acad. Sci. USA* **80**: 4803 (1983). The hygromycin phosphotransferase gene, which confers resistance to the antibiotic hygromycin, is a further example of a useful selectable marker. Vanden Elzen *et al.*, *Plan Mol. Biol.* **5**: 299 (1985). Additional positive selectable markers genes of bacterial origin that confer resistance to antibiotics include gentamicin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase and the bleomycin resistance determinant. Hayford *et al.*, *Plant Physiol.* **86**: 1216 (1988); Jones *et al.*, *Mol. Gen. Genet.* **210**: 86 (1987); Svab *et al.*, *Plant Mol. Biol.* **14**: 197 (1990); Hille *et al.*, *loc. cit.* **7**: 171 (1986).

Other positive selectable marker genes for plant transformation are not of bacterial origin. These genes include mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase and plant acetolactate synthase. Eichholtz *et al.*, *Somatic Cell*

Mol. Genet. **13**: 67 (1987); Shah *et al.*, *Science* **233**: 478 (1986); Charest *et al.*, *Plant Cell Rep.* **8**: 643 (1990).

Another class of useful marker genes for plant transformation with the DNA sequence requires screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. These genes are particularly useful to quantitate or visualize the spatial pattern of expression of the DNA sequence in specific tissues and are frequently referred to as reporter genes because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression. Commonly used genes for screening presumptively transformed cells include β-glucuronidase (GUS), β-galactosidase, luciferase, and chloramphenicol acetyltransferase. Jefferson, *Plant Mol. Biol. Rep.* **5**: 387 (1987); Teeri *et al.*, *EMBO J.* **8**: 343 (1989); Koncz *et al.*, *Proc. Nat'l Acad. Sci. USA* **84**: 131 (1987); De Block *et al.*, *EMBO J.* **3**: 1681 (1984). Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig *et al.*, *Science* **247**: 449 (1990).

In order to create an expression vector containing the gene or DNA segment of interest, an expression cassette first is made by inserting a cloned *f12* gene, or a DNA segment comprising the *f12* signal sequence fused to a desired high-value protein as described above, into a plasmid under the control of a regulatory sequence. The resulting expression cassette can be ligated back to itself to produce an expression cassette with a tandem repeat of the cloned gene. A further ligation can be performed to generate a construct that contains four tandem copies of the gene.

One or more copies of the expression cassette containing the introduced DNA segment corresponding to the *f12* gene or to the DNA segment comprising the *f12*

signal sequence fused to a desired protein is transferred to an expression vector. In a preferred embodiment, the vector also contains a gene encoding a selection marker which is functionally linked to promoters that control transcription initiation.

To create a transgenic plant, an expression vector containing the *f12* gene or the DNA segment comprising the *f12* signal sequence fused to a desired protein can be introduced into protoplasts; into intact tissues, such as immature embryos and meristems; into callus cultures or into isolated cells. Preferably, expression vectors are inserted into intact tissues, such as explants derived from hypocotyl or cotyledonary nodes of a germinated seed. (In this regard, an explant is a piece of tissue that is taken from a donor plant and is capable of producing callus in culture. Hypocotyl tissue is that portion of the stem of a plant embryo or seedling below the cotyledons and above the root. A cotyledon is an embryonic leaf, and a cotyledonary node is that part of the seedling between the embryonic axis and the cotyledons which botanically defines the division of the hypocotyl and the epicotyl, or embryonic shoot.) General methods of culturing plant tissues are provided, for example, by Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," *in* METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY 67-88 (CRC Press 1993).

Preferably, the *f12* gene or DNA segment comprising the *f12* signal sequence fused to a desired high-value protein is transformed into embryogenic maize callus by particle bombardment. Transgenic maize plants are produced by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids. The plasmids consist of a selectable and an unselectable marker gene.

The present invention is described further by reference to the following, illustrative examples.

Example 1. *Characterization of 24-kDa protein of fl2*

Wild-type maize, W64A+, was crossed with W64A*fl2* to give an F2 population, according to Lopes *et al.*, 1994, the contents of which are incorporated herein by reference. The progeny were sorted into floury, semi-floury and normal kernels, and DNA was isolated from 30 individuals in each phenotypic class. To identify DNA polymorphisms linked with the *fl2* mutation, a bulked segregant analysis was used. Michelmore *et al.*, *Proc. Nat'l Acad. USA* **88**: 9828-32 (1991). After extensive screening with different DNA probe/restriction enzyme combinations, an RFLP was detected in SstI-digested DNA with a 22-kDa α-zein cDNA. A 7.7 kb DNA fragment was present in the homozygous *fl2* bulked sample and the heterozygous semi-floury bulk, but not in the normal bulk.

The 7.7 kb SstI fragment was isolated from genomic DNA of W64A*fl2*, and the resulting clone, pCC515, was mapped by restriction enzyme digestion, as described in Coleman *et al.*, *Proc. Nat'l Acad. Sci. USA* **92**: 6828-31 (1995), the contents of which are incorporated herein by reference. Clone pCC515 was found to contain a single 22-kDa α-zein coding sequence, which was obtained as a 1.6 kb EcoR1 fragment. Upon nucleotide sequence analysis, the deduced amino acid sequence was found to correspond to a 22-kDa α-zein. The protein contains 262 amino acids, including a 21 amino acid signal peptide.

Comparison of the deduced amino acid sequence of the signal peptide with the signal peptides of other α-zeins reveals an alanine to valine substitution at the C-terminal (-1) residue of the signal peptide, insertion of a histidine following the seventh residue in the seventh α-helical repeat and an alanine to thoureonine substitution in the same α-helical repeat. N-terminal sequence analysis of the purified 24-kDa protein from *fl2* endosperm showed an identical match for the first 45 amino acid residues between pCC515 and the 24-kDa polypeptide. The signal peptide that targets the protein

into the lumen of the rough endoplasmic reticulum was found to be attached to the protein.

Example 2. ***Transformation of maize with the f12 gene***

To prove that pCC515 contains the *f12* gene, the gene was transformed into embryogenic maize callus by particle bombardment. Transgenic maize plants were produced by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids. The plasmids consist of a selectable and an unselectable marker gene.

Preparation of tissue

Immature embryos of maize variety High Type II were the target for particle bombardment-mediated transformation. This genotype is the F_1 of two purebred genetic lines, parents A and B, derived from the cross of two know maize inbreds, A188 and B73. Both parents were selected for high competence of somatic embryogenesis, according to Armstrong *et al.*, *Maize Genetics Coop. News* **65**: 92 (1991). The High Type II genotype does not possess the native mutant *f12* gene.

Ears from F_1 plants were selfed or sibbed, and embryos were aseptically dissected from developing caryopses when the scutellum first became opaque. This stage occurred about 9-13 days post-pollination, and most generally about 10 days post-pollination, depending on growth conditions. The embryos were about 0.75 to 1.5 millimeters long. Ears were surface sterilized with 20-50% Clorox for 30 minutes, followed by three rinses with sterile distilled water.

Immature embryos were cultured with the scutellum oriented upward, on embryogenic induction medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCL, 30 gm/l sucrose, 2.88 gm/l L-proline, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, and 8.5 mg/l $AgNo_3$. Chu *et al.*, *Sci. Sin.* **18**: 659 (1975); Eriksson, *Physiol. Plant* **18**: 976 (1965). The medium was sterilized by autoclaving at 121°C for 15

minutes and dispensed into 100 X 25 mm Petri dishes. $AgNO_3$ is filter-sterilized and added to the medium after autoclaving. The tissues were cultured in complete darkness at 28°C. After about 3 to 7 days, most usually about 4 days, the scutellum of the embryo had swelled to about double its original size and the protuberances at the coleorhizal surface of the scutellum indicated the inception of embryogenic tissue. Up to 100% of the embryos displayed this response, but most commonly, the embryogenic response frequency was about 80%.

When the embryogenic response was observed, the embryos were transferred to a medium comprised of induction medium modified to contain 120 gm/l sucrose. The embryos were oriented with the coleorhizal pole, the embryogenically responsive tissue, upwards from the culture medium. Ten embryos per Petri dish were located in the center of a Petri dish in an area about 2 cm in diameter. The embryos were maintained on this medium for 3-16 hour, preferably 4 hours, in complete darkness at 28°C just prior to bombardment with particles associated with plasmid DNAs containing the selectable and unselectable marker genes.

To effect particle bombardment of embryos, the particle-DNA agglomerates were accelerated using a DuPont PDS-1000 particle acceleration device. The particle-DNA agglomeration was briefly sonicated and 10 μl were deposited on macrocarriers and the ethanol was allowed to evaporate. The macrocarrier was accelerated onto a stainless-steel stopping screen by the rupture of a polymer diaphragm (rupture disk). Rupture was effected by pressurized helium. The velocity of particle-DNA acceleration was determined based on the rupture disk breaking pressure. Rupture disk pressures of 200 to 1800 psi were used, with 650 to 1100 psi being preferred, and about 900 psi being most highly preferred. Multiple disks were used to effect a range of rupture pressures.

The shelf containing the plate with embryos was placed 5.1 cm below the bottom of the macrocarrier

platform (shelf #3). To effect particle bombardment of cultured immature embryos, a rupture disk and a macrocarrier with dried particle-DNA agglomerates were installed in the device. The He pressure delivered to the device was adjusted to 200 psi above the rupture disk breaking pressure. A Petri dish with the target embryos was placed into the vacuum chamber and located in the projected path of accelerated particles. A vacuum was created in the chamber, preferably about 28 in Hg. After operation of the device, the vacuum was released and the Petri dish was removed.

Bombarded embryos remained on the osmotically-adjusted medium during bombardment, and 1 to 4 days subsequently. The embryos were transferred to selection medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCL, 30 gm/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, 0.85 mg/l Ag NO_3 and 3 mg/l bialaphos (Herbiace, Meiji). Bialaphos was added filter-sterilized. The embryos were subcultured to fresh selection medium at 10 to 14 day intervals. After about 7 weeks, embryogenic tissue, putatively transformed for both selectable and unselected marker genes, proliferated from about 7% of the bombarded embryos. Putative transgenic tissue was rescued, and that tissue derived from individual embryos was considered to be an event and was propagated independently on selection medium. Two cycles of clonal propagation were achieved by visual selection for the smallest contiguous fragments of organized embryogenic tissue.

A sample of tissue from each event was processed to recover DNA. The DNA was restricted with a restriction endonuclease and probed with primer sequences designed to amplify DNA sequences overlapping the *f12* and non-*f12* portion of the plasmid. Embryogenic tissue with amplifiable sequence was advanced to plant regeneration.

For regeneration of transgenic plants, embryogenic tissue was subcultured to a medium comprising MS salts

and vitamins (Murashige & Skoog, *Physiol. Plant* **15**: 473 (1962)), 100 mg/l myo-inositol, 60 gm/l sucrose, 3 gm/l Gelrite, 0.5 mg/l zeatin, 1 mg/l indole-3-acetic acid, 26.4 ng/l ±cis-trans-abscissic acid, and 3 mg/l bialaphos in 100 X 25 mm Petri dishes, and was incubated in darkness at 28°C until the development of well-formed, matured somatic embryos could be seen. This required about 14 days. Well-formed somatic embryos were opaque and cream-colored, and were comprised of an identifiable scutellum and coleoptile. The embryos were individually subcultured to a germination medium comprising MS salts and vitamins, 100 mg/l myo-inositol, 40 gm/l sucrose and 1.5 gm/l Gelrite in 100 X 25 mm Petri dishes and incubated under a 16 hour light:8 hour dark photoperiod and 40 μeinsteinsm^{-2}sec^{-1} from cool-white fluorescent tubes. After about 7 days, the somatic embryos had germinated and produced a well-defined shoot and root. The individual plants were subcultured to germination medium in 125 X 25 mm glass tubes to allow further plant development. The plants were maintained under a 16 hour light:8 hour dark photoperiod and 40 μeinsteinsm^{-2}sec^{-1} from cool-white fluorescent tubes. After about 7 days, the plants were well-established and were transplanted to horticultural soil, hardened off, and potted into commercial greenhouse soil mixture and grown to sexual maturity in a greenhouse. An elite inbred line was used as a male to pollinate regenerated transgenic plants.

Preparation of particles

Fifteen mg of tungsten particles (General Electric), 0.5 to 1.8 μ, preferably 1 to 1.8 μ, and most preferably 1 μ, were added to 2 ml of concentrated nitric acid. This suspension was sonicated at 0°C for 20 minutes (Branson Sonifier Model 450, 40% output, constant duty cycle). Tungsten particles were pelleted by centrifugation at 10000 rpm (Biofuge) for one minute, and the supernatant was removed. Two milliliters of sterile distilled water were added to the pellet, and brief sonication was used to resuspend the particles. The

suspension was pelleted, one milliliter of absolute ethanol was added to the pellet, and brief sonication was used to resuspend the particles. Rinsing, pelleting, and resuspending of the particles was performed two more times with sterile distilled water, and finally the particles were resuspended in two milliliters of sterile distilled water. The particles were subdivided into 250-μl aliquots and stored frozen.

Preparation of particle-plasmid DNA association

The stock of tungsten particles was sonicated briefly in a water bath sonicator (Branson Sonifier Model 450, 20% output, constant duty cycle) and 50 μl was transferred to a microfuge tube. Equimolar amounts of selectable and unselectable plasmid DNA were added to the particles for a final DNA amount of 0.1 to 10 μg in 10 μl total volume, and briefly sonicated. Preferably, 1 μg total DNA was used. Specifically, 3.5 μl of DP4810 (ubi_p:ubiint::BAR::$pinII_t$, 5.6 kbp) plus 6.5 μl of DP6645 ($fl2_p$::fl2::$fl2_t$, 10.2 kbp), both at 0.1 μg/μl in TE buffer, were added to the particle suspension. Fifty microliters of sterile aqueous 2.5 M $CaCl_2$ were added, and the mixture was briefly sonicated and vortexed. Twenty microliters of sterile aqueous 0.1 M spermidine were added and the mixture was briefly sonicated and vortexed. The mixture was incubated at room temperature for 20 minutes with intermittent brief sonication. The particle suspension was centrifuged, and the supernatant was removed. Two hundred fifty microliters of absolute ethanol were added to the pellet, followed by brief sonication. The suspension was pelleted, the supernatant was removed, and 60 μl of absolute ethanol were added. The suspension was sonicated briefly before loading the particle-DNA agglomeration onto macrocarriers.

Example 3. ***Extraction and characterization of protein from transgenic seed***

Embryos were hand-dissected from dry, mature kernels sampled from fully developed ears and endosperms were

pulverized to a fine meal with a ball mill. Alpha-zeins were extracted overnight in 70% (v/v) ethanol with constant shaking at 37°C. After centrifugation for 15 minutes at 12,000 rpm, the supernatant was collected, vacuum dried, and stored at 4°C until use. Total zeins and non-zein proteins were isolated according to Wallace *et al.*, *Plant Physiol.* **92**: 191-96 (1990).

SDS-polyacrylamide gels (10 and 12.5%, w/v) and gradient gels (7.5-18%, w/v) were prepared according to Laemmli, *Nature* **227**: 680-85 (1970), but the TRIS concentrations used in the resolving gel and running buffer were doubled. Protein samples were diluted in Laemmli sample buffer and boiled for 3 minutes before loading. Gradient and 12.5% gels were run at room temperature at a constant current until the dye front migrated throught the stacking gel, and then at 250 mA through the resolving gel. Gels were stained with Coomassie overnight, and destained in 40% (v/v) methanol and 10% (v/v) acetic acid for at least 8 hours. Immunoblotting analyses were used specifically to detect α-zeins in protein extracts. Protein extracts were separated by SDS-PAGE as described above, transferred to nitrocellulose filters, and treated with a rabbit anti-α-zein polyclonal antibody. Lending *et al.*, *Protoplasma* **143**: 51-62 (1988). Goat anti-rabbit alkaline phsophatase conjugate was used for indirect detection of α-zein, as described by Knecht *et al.*, *Anal. Biochem.* **136**: 180-84 (1984). The 24 kDa α-zein was detected in the seed from the transgenic plants.

WHAT IS CLAIMED IS:

1. A cereal plant that contains a transgene comprised of (i) a first polynucleotide segment comprising a nucleotide sequence that encodes the amino acid sequence MATKILALLALLALLVSATNV and (ii) a second polynucleotide segment coding for a protein.

2. A cereal plant as claimed in claim 1, wherein said first and second polynucleotide segments are operably linked to a promoter, such that said cereal plant expresses both segments under the control of said promoter.

3. A cereal plant as claimed in claim 2, wherein said promoter is the *fl2* promoter.

4. A cereal plant as claimed in claim 1, wherein said plant is a maize plant and wherein said segment (ii) is not native to maize.

5. A cereal plant as claimed in claim 1, wherein said plant is rice, wheat, barley, millet or sorghum.

6. A cereal plant as claimed in claim 5, wherein said plant is rice or wheat.

7. A cereal plant as claimed in claim 1, in which said polynucleotide segment (ii) has a high content of an amino acid selected from the group consisting of methionine, lysine, tryptophan and threonine such that the amount of said amino acid in seeds of said cereal plant is increased as compared to seeds from otherwise identical plants that are not transformed.

8. A seed produced by a plant as claimed in claim 1.

9. A feed product comprising meal obtained from seed as claimed in claim 8.

10. A transgene comprised of (i) a first polynucleotide segment comprising the nucleotide sequence coding for the amino acid sequence MATKILALLALLALLVSATNV and (ii) a second polynucleotide segment coding for a protein.

11. A transgene as claimed in claim 10, wherein said first and second polynucleotide segments are operably linked to a promoter.

12. A transgene as claimed in claim 11, wherein said promoter is the *fl2* promoter.

13. A transgene as claimed in claim 10, comprising the coding region of *fl2*.

14. A transgene as claimed in claim 10, additionally comprising a sequence of *fl2* selected from nucleotides 761-3824 of Figure 1.

15. A transgene as claimed in claim 10, additionally comprising a sequence of *fl2* selected from nucleotides 4613-8335 of Figure 1.

16. A transgene as claimed in claim 14, additionally comprising a sequence of *fl2* selected from nucleotides 4613-8335 of Figure 1.

17. A transgene comprising the *fl2* promoter operably linked to a polynucleotide segment.

FIGURE 1A

1 CTAAATTGTA AGCGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT

51 AAATCAGCTC ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT

101 AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA

151 CAAGAGTCCA CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA

201 CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC CTAATCAAGT

251 TTTTTGGGGT CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG

301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG

351 AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG

401 GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA

451 GGGCGCGTCC CATTCGCCAT TCAGGCTGCG CAACTGTTGG GAAGGGCGAT

501 CGGTGCGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT

551 GCAAGGCGAT TAAGTTGGGT AACGCCAGGG TTTTCCCAGT CACGACGTTG

601 TAAAACGACG GCCAGTGAGC GCGCGTAATA CGACTCACTA TAGGGCGAAT

651 TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATATC

701 GAATTCCTGC AGCCCGGGGG ATCCACTAGT TCTAGAGCGG CCGCCACCGC

751 GGTGGAGCTC ATGCAATTTT AGCCAAATAT CATTAGCAGT TTTCAGGGTA

801 AATACTTGGT TGAAAATATC CATGCTAAGA GATTCATACA AGCAATTTTT

851 GGCTCTAGCA TTTAAATGAA TTTCTTTTTC CTCACTCGTG GTGGGTTTCT

901 CGGGATTCTT GAGAGGTTTC ATCCCGTCAC GAGTGACTCT CCAAACACCT

951 AGATCAACGG CCTCTAGGTA ACAAGCCATT CTAGCACTAT AATATGGGAA

1001 GTTAGTGCCG TCGAAGTGTG GTGGCCTATG TGTATCCATC CCTTCCTCTA

1051 AAAAGCGTCG GCTCTTTTAG CGGTGAAGCT AAAGCGTTTC AAATGAGCCA

1101 AACCGGGCTC TGATACCAAT TGTAGGAAAC GGGTGACGCC TAAGAGGGGG

1151 GGGGGTGAAT TAGGACTTCT AAAACTTTTA CTAAAACTAG GCCACAATTA

1201 AATCCCTAGA GCAAAACCTA TGCAAGTAAT CAAAACTACA ATGTGCAAAC

1251 TAGGTTTTGT CTAAGTGTTG CTATCTCTAC CGCAAAGGCT AAGTTTCAAT

1301 CTACACTAAA TAAGTATGAC AATAAGATTG AAACTTAAAT GCTTAATATA

FIGURE 1B

1351 AATGTGGAAT GTAAAGCGCT AAGTAGAGAA GCAAACTCTT GTGGATGACG

1401 CCGGTATTTT TACCGAGGTA TCCGGAACCG CGCAAGGTCC CGACTAATCC

1451 TCGTTGGTGC CCCTACGCAA AGGGAAGCCC ACGCGAGGGC CAAGCACCAC

1501 GGTCGAGTAA CTCCGTAGAG AGCCGCGGGC CTTCTCCACG CGCAAGTGGT

1551 GCTCCGCTTC CGGCTCCTCT CGGACGCTCC CCGCCGTCTC CACTATCGAG

1601 CTTCCGGTCG AAACGCCGCG GGCCTCGTTC CCTCCGGTAC ACGGTGGCGG

1651 CCGTGACACA AACGCGGTTG TCACGGTCTC GCAAGACTCT CGCCCCACTC

1701 GGTACAATTA CAACGACTCA CGCAAGAGCC GAGGGGTTGT GAGGTTTATC

1751 TAAACTCACT CAACTAACTA GGATTCACCT AGAGCAAGCG CTAAAGCGGT

1801 CTAACTAACC TAAGCACTTC GCAAAGCACC TACGCTAATC ACCGAGTGAT

1851 TCTATTAAGC ACTTGGGTGT TTGAGCACTT GGAGATATGC ACTATGTGTA

1901 TTGGAATGTT GCTTGGGCTC TCACACTAGA GAATGGCCGG TTGGGGTGGT

1951 ATTTATAGCC TCCACACCCC CAACTAGCCG TTGGACAGAA AGCAGCAGCT

2001 TTCTGTCGTC GGGTGCACCG GACAGTCCGG TGCACCACTG GACACTGAAC

2051 AGTAGATGTC CGGTGCACGC CACGTCAGCC GACCGTTGGC GCCTGTAGCA

2101 GTCGACCGTT GGATCCGACC GTTGCCTTCT GCCCGTTGGC ACACCGGACA

2151 GTCCGGTGCA CACCGGACAG TCCGGTGCTA CAGCCAGAGA GCGCCTGTCT

2201 GCGGCCTCTC TGCGCCGACT GTCCGGTGCA CACCGGACAC CGATGTCCGG

2251 TGCGCCACCA GGCGCTGGCT GACAGCCCTT GTCTTGGATT TCTTCGCTGA

2301 TTTCTTCGGG CTTCTTTGTT CTTGAGTATT GGACTCCTAT GCATCTTTTT

2351 ATGTCTTCTT TTGAGGTGTT GCATCCTCAT TGCCTTGGTC CAATTCTCTT

2401 CGCATCCTGT GAACTACAAA CACAAACACT AGAAGACTTA TTAGTTCACT

2451 GATTGTGTTG TTCATCAAAC ACCAAAACTC AATTAGCCAA ATGGCCCGGG

2501 GTCCATTTTC CTTACAACTT CAACGGCCGC ACCGACCCTC TGACCTCTCC

2551 TTTTCTCTCC TTTCTCACTC CTATCGGTAG CTACAACAGA AGCGACCCCC

2601 AACGCGGCGC AAACCCTCGA AGCATACGGC TGGGGAAGAC GGCAGCCAGG

2651 TTTATATCCT AGGCGCCCGA GGAAATCGCG CGGTCAGCTG TTACGGTTCG

FIGURE 1C

2701 CCCGCGGGGC ACGATTCGCG CGAAGAAGAC CGTATGCAAG GGAGGGCCCA

2751 CTAGCAGCGA GCCATCACCT AGGGAAGCGT GCATGCATCG ATTGACACGC

2801 GACCCCAACA GTCAGGCGAC CCGAGTGTGC AGACGGTCGT GATGGTGAAA

2851 GTGGCCGGCC CGCGCGGACG CGTAGGGGCA TTGGGCCAAA ATGCGTTTCA

2901 GCGGCCCAGC TTCTTTTTTC TTCTATTTTT TTCTTTCCTT TTCCTTTCTA

2951 TTTTTAGATT TCAAATTTAA GTTCAAATTT TTTTTATGGT GAATTTTCTA

3001 AAAATCCGCA GACTAGTATG AAAAGAATTT ATATATAAAT CTATTTATTT

3051 ATATATTTAT TTTCTATGTT ATTTCCAATT TCTAAAATGT AAATTAGGTT

3101 AAATCGCCAT TTGGACACTA ATATATCTTT ATTAGTATTA CTATTATTAG

3151 ATGCACAACC AAATAAACTC CAACATGATG CATCGATTAT TTGTATGCCA

3201 TTGGTTAATT ATTCACTTTA AATATGCTCC TTAACGATTC TCATGAAACA

3251 GAAGGCCATG CACATAAAGA TGTATCCCTT TCTTTTATAT TCCCAGAGTT

3301 GGGTATTACA ACATTCATCT ATGCATTCTA GGATTTCAAT TAATCTCAAT

3351 CTTTTAGTAT TTGTTCCTTC ATTCTCAAAT CACTTCTCAT CTAACTACTA

3401 TGCTTGTTTA ACCAGCACAA CAATACTACA ACAATATCCA TTTATAAAGG

3451 CTTTAATAGC AAACTTTACA TATTCATATC ATGTTAAGGT TGTCACATGT

3501 GTAAAGGTGA AGAGATCATG CGTGTCATTC CACATAATGA AAAGAATTCC

3551 TATATAAAAA CGACATGTTT TGTTGTAGGT AGTGGAAACT ATCTTTCCAG

3601 CAAAGACCAT ATAATCCGAT AAAGCTGATA ACTAAATGTC GAAATCGAGT

3651 AGGTGCCATA TCATCTATAT CTTATCTGTT GTTTGGAAAA AGACAAAATC

3701 CAAAAAAAAT ATATGAGATC TCACCTGTAT AAATAGCTCC CAAATCAGTA

3751 GTTAATACAT CTCCCATAAT ATTTTCAGCA TTCAGAAACA CACCAAGCGA

3801 ACGACTAGCA ACGACCTAAC AACAATGGCT ACCAAGATAT TAGCCCTCCT

3851 TGCGCTTCTT GCCCTTTTAG TGAGCGCAAC AAATGTGTTC ATTATTCCAC

3901 AGTGCTCACT TGCTCCTAGT GCCATTATTC CACAGTTCCT CCCACCAGTT

3951 ACTTCAATGG GCTTCGAACA TCCAGCCGTG CAAGCCTATA GGCTACAACT

4001 AGTGCTTGCG GCGAGCGCCT TACAACAACC AATTGCCCAA TTGCAACAAC

FIGURE 1D

4051 AATCCTTGGC ACATCTAACC CTACAAACCA TCGCAACGCA ACAACAACAA

4101 CATTTTCTGC CATCACTGAG CCACCTAGCA GTGGTGAACC CTGTCGCCTA

4151 CTTGCAACAG CAGCTGCTTG CATCCAACCC ACTTGCTCTG GCGAACGTAG

4201 CTACATACCA GCAACAACAA CAGCTGCAAC AGTTTATGCC AGCGCTCAGT

4251 CAACTAGCCA TGGTGAACCC TGCCGTCTAC CTACAACTGC TTTCATCTAG

4301 CCCGCTCGCT GTGGGCAATG CACCTACGTA CCTACAACAA CAGTTGCTGC

4351 AACAGATTGT ACCAGCTCTA ACTCATCAGC TAGCTATGGC AAACCCTGCT

4401 ACCTACTTAC AACAGTTGCT TCCATTCAAC CAATTGGCTG TGTCGAACTC

4451 TGCTGCGTAC CTACAACAGC GACAACAATT ACTTAATCCA TTGGCAGTGG

4501 CTAACCCATT GGTCGCTACC TTCCTGCAGC AGCAACAATT GCTGCCATAC

4551 AACCAGTTCT CTTTGATGAA CCCTGCCTTG CAGCAACCCA TCGTTGGAGG

4601 TGCCATCTTT TAGATTACAT ATGAGATGTA CTCGACAATG GTGCCCTCAT

4651 ACCGGCATGT GTTTCCTAGA AATAATCAAT ATATTGATTG AGATTTATCT

4701 CGATATATTT CTGAACTATG TTCATCATAT AAATAACTGA AAACATCAAA

4751 TCGTAATTTT AAAGCTCATG CTTGGTCAAT ACATAGATAA TACAATATTA

4801 CTTCATCATC CCAATGATGT CCTAGCACAA CCTATTGAAT GTTAATGTTT

4851 GGTTGTGTGG GGGTGTGTTT ATAACATAGA TGTGATTATT TGTGCTTTTT

4901 GTTGAGTATA TACATATATG GTATGTTGAT TTGATATAGT GATGGACACA

4951 TGCTTTGGCC TTGGATATTC AAATCACTTG TACTTGCACG AAGCAAAACA

5001 TAATATAAGT TTAGAAGTAA ACTTGTAACT GTGTCCAAAC ATGCTCACAC

5051 AAAGTCATAT CGCATTATAT TTTTTTGGTA AATATTCAAC ACATGTATTT

5101 TTTACAAGAA CCCAAATTTT ACAGACAAAT GCAGCATTGT AGACATGTAG

5151 AATTCTTTGA AGCATGTGAA CTTAACAACA CTAATGTCAT TAAATCAACT

5201 AGTAACAATT TCGATATTGC AAACACCAAA TTATGGAACT

5251 TATTTGCTGA AAAAATTATG ATCAATGTGA AGTTTAAATT ATTATACCAT

5301 AAATATATCA AAGATTTTTT TTGAGGAAGG TAAAAATTGC ATGGAATGGG

5351 CTGCCCAACG TGATAGCTCA CTTTTATGCT AGGTAGCATT ACCAAAGATG

FIGURE 1E

5401 GGAATGTTCT GATGAACACC AAACCCACTC AAATAATATT TATATTTGGG
5451 TTGTTTAGTT GTAAAAGTGA AGACCCAAGA TTAAAGTACC AATTGGCCAA
5501 TGACATTCGA TTGTTTTGTT CAAAGAGCAC TTGGTGCGTC ATTTGGACTC
5551 GTATCTTAGT CCAATAGATT GCATTTTCCT TCAATGTGTA GAATCCGACA
5601 AAGTGCATGT TCTAAAATTG TAAATCTAAC TAAATTAGAA AGTTTGTTAC
5651 TAATTTGATG GGTTTATTAG GTGTAGCTCA TAAAACTATA ACCAACATAC
5701 CCTGCTCTCA CATGTCATAG AAAATAGGAG TATCCAAGCA TAATTTGTGT
5751 GAGCATCCAT GACACAAATT ATATATAGAC TGATACAATT AATTCCTTCA
5801 AAAATAAAAA AATAAAAACC AAAAAGTGTT TTTAATACTG CTGGATTCTT
5851 CTTTAGCTAA TCAGGAGTAC TAAAAAGATG TTGCACTTTT GGGTGGCGAG
5901 AATTCCTCAG TCATAACTTT GACATAATTT TAATCCGGAT CACATATATA
5951 TCATCATATA TTAATTATTA AATTAATATG GTATGCATCA TCGATTTATT
6001 AAATCAATCT GGTATAACCA TCGGATCTTA ATTATTAAAT TATTCTAGAG
6051 CGTACATTAG ATTATCAAAT CAAATCAACC CGACATAACC ATCAAATGTT
6101 CCGCTATGCA CCATAAGCGC ATGGTCTGGG GCTTAATCAT CCGGAATAAC
6151 CATCCGATCT TCCGGTAAGC ATAGTAAGCG TATGGTTCGG GGCTTCGTCA
6201 TCCAGAATAA CCATTAGATT TTCCGGTATG CACTGAAAGA CTATGATCCA
6251 GGGTTATGAT AGGTGAAACG TTGTTAGGTT ATTGTGGATG CTAGCTGCTG
6301 CCGCGTGTGC GATCTGTTGT GTGGAAGCTG CTTGGTCCTC TTGTGTTGCA
6351 CGTGGCTTGT GGTGGGCTTG GACTACTCTC ATGGCTTCAG CGCTTGGAGC
6401 TGCTCGTCTA TCCAGTCCTG CGTGCGTCTC TCTCTCCTGG CTGGAGGCGT
6451 GGTGGCTAGG CGACTTCTCT GTGTTTTTCT TTTTGCGGCG GCTGCTCGTG
6501 TTGGCGCCAG GGGCCCGATT AGGTATGCTC CCTCAGCTTT TATAGGTAGC
6551 GCAACACAGT AGGCAGAGCA AAGATAAAGA TTCCAACTGC ATCCATCTAC
6601 AAACAAGGCT CTCGGTGGAA GGGATAAAAG CATGCAGCGC AATTGCCGCT
6651 GCCCTGCTGC AACCTTGACT CTCGTCAATT GGATGACCAA AAGAGGCCAC
6701 CGTCTAATCT CTTTTCTTTG TGCAGCTACC TGTTTGTCAC GAACACAAAA

FIGURE 1F

6751 TTGCAGGTGC CTATTTGTGC AGGTGCCTGT TTGTCACCAA TTATCCTCTA

6801 CCACAAAGAA CTACAATAGT GATGGAGGAT CCGATCCGGC ATAGATCCAC

6851 TATAATGCAT AGTCGGTGAT GCATGCCTAA TTGTGGTTGT TTAGCGTTAG

6901 TTTGGGCATG TTGGAACTAA GCAATTGTCG ATGCCATACA CTAGAAGTGA

6951 AGAATGTTGT TAGCGTTAGG AGTAGATGTG TCAATGGGGA CCGATACTCG

7001 TTAACCCATG GAGAATTTCT CTATTAGATT TAGGGTATGT GAAGATTTTA

7051 ATCTCCATTT GCTGAAGGTA GGCATACCGC ATACATCTAT TTGAATCAAC

7101 AGTTACAGTT CAACACATAC TCCTTTCGCA CTAGTTTGTT GCGTGTCCTA

7151 CCTTTTTCCT AAATCGGTTT TCCCAAAATT GACATACTCC CTAGAAAATA

7201 CATCCAAATT TTGAAGATCA AATTTGTTCC ATTGAATTAT TCATGACATA

7251 TATACCGATA TTGCTTTTTC ATCATTAGAA TATAATTTTG AACTCAGTAA

7301 AAGTTACATA GGATTCTTTT TAGACAATAA TAAAGCAAAT TGTAATCAAA

7351 ACACTTTCAT CATAGGGATT TATCCTGAGG TTAGGCCAAA CATGAAATGC

7401 TTGCCTAGTC CTCATTGGAG TTAGCCACAC CTTGGCTTAG AGTCTATTTC

7451 AACTCTTTCC TCCGTTTGCT CGGATCTATC AAAGCGATAG ATAGAGCCTT

7501 CCTCTATGTC GATGAAGGTT ACAACGATGT CGTTACTGCT TACAGTCTTC

7551 TTGACAGCAC TCCAGCAAAG TAACACCTTG CTCAAGATCT TGCTCTAGCT

7601 CTCAGTACCA CTTCCCCTCT CTCTAAAACC TTATAAATTT GCCTCTACAC

7651 AAACTAGAGA GATACATAAG AGAGGGAGAG AAAAAATTTG ATCACTTGAT

7701 GTATGGACTT GTTTTGTGAC CTACAAATGG GCGCCTAGGG GTCCCTTTAT

7751 AGTCTCAAGG GAGCCCTAGC TGTTGCCTCT TTCAAATAGA AGTTGCTAGA

7801 AAAGTTTCCC TGGTTGTGGG GGCACCGGAC TGTTCGGTGT GCTTTCATCC

7851 AACGATCAGC GAATCCTTAA TTGGCCACCT TCCTCTTTTG TAGGGCACCG

7901 AATTGTCCGG TGCTAGCACC GGACTATCCG GAGTTCCAAT TTGCCCATTG

7951 GCGCCTGCTG ACGTGGCCAA CTAGCAGTTG CGCGAAGACA ATCAGAGTGT

8001 CCGGTGACAG GGCTTGGACT ATCTAGTGCA TGGTCCCGTA CTGTCCGGTG

8051 TTTTTAGCCG AGATACCCGA GTTGGGCAGT TGGTCGCCCA GGACATTGGA

FIGURE 1G

8101 CTATCCGGTG CACACTGGAC TGTCTGGTGA GGTGCACCGG ACTATCTGGT

8151 GCTACCCAGA CAACAACACT CTTATGTGCT TTTCCTCCTT TTCTTCTCCA

8201 TTCAAATCTT GGGAGGGTCT TCCTGTGATT TAGACAAACA TAGTTAGAGA

8251 CTTAGAGTAC ACATCAATTC ACTCAGTCCT AGAATTAACT CTTTTCAAAT

8301 CTTCTCCCAA CTTTTCTATT TCACCTCAAA TAGAGCTCCA GCTTTTGTTC

8351 CCTTTAGTGA GGGTTAATTG CGCGCTTGGC GTAATCATGG TCATAGCTGT

8401 TTCCTGTGTG AAATTGTTAT CCGCTCACAA TTCCACACAA CATACGAGCC

8451 GGAAGCATAA AGTGTAAAGC CTGGGGTGCC TAATGAGTGA GCTAACTCAC

8501 ATTAATTGCG TTGCGCTCAC TGCCCGCTTT CCAGTCGGGA AACCTGTCGT

8551 GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG CGGTTTGCGT

8601 ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT

8651 TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT

8701 CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG

8751 CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG

8801 GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT

8851 GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC

8901 TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC

8951 CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA

9001 GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC

9051 GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT

9101 TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG

9151 GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG

9201 AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG

9251 CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT

9301 CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

9351 CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC

9401 TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG

FIGURE 1H

9451 TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA

9501 TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG

9551 TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC

9601 GTTCATCCAT AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG

9651 GAGGGCTTAC CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG

9701 CTCACCGGCT CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG

9751 AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT

9801 TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA

9851 CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA

9901 TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC

9951 CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT

10001 CAGAAGTAAG TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC

10051 ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT

10101 GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG

10151 CTCTTGCCCG GCGTCAATAC GGGATAATAC CGCGCCACAT AGCAGAACTT

10201 TAAAAGTGCT CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG

10251 ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA

10301 CTGATCTTCA GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA

10351 CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT

10401 TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG

10451 TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC

10501 AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCAC

FIGURE 2

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-21   M  A  T  K  I  L  A  L  L  A  L  L  A  L  L  V  S  A  T  N
      ATG GCT ACC AAG ATA TTA GCC CTC CTT GCG CTT CTT GCC CTT TTA GTG AGC GCA ACA AAT

-1    V  F  I  I  P  Q  C  S  L  A  P  S  A  I  I  P  Q  F  L  P
      GTG TTC ATT ATT CCA CAG TGC TCA CTT GCT CCT AGT GCC ATT ATT CCA CAG TTC CTC CCA

20    P  V  T  S  M  G  F  E  H  P  A  V  Q  A  Y  R  L  Q  L  V
      CCA GTT ACT TCA ATG GGC TTC GAA CAT CCA GCC GTG CAA GCC TAT AGG CTA CAA CTA GTG

40    L  A  A  S  A  L  Q  Q  P  I  A  Q  L  Q  Q  Q  S  L  A  H
      CTT GCG GCG AGC GCC TTA CAA CAA CCA ATT GCC CAA TTG CAA CAA CAA TCC TTG GCA CAT

60    L  T  L  Q  T  I  A  T  Q  Q  Q  Q  H  F  L  P  S  L  S  H
      CTA ACC CTA CAA ACC ATC GCA ACG CAA CAA CAA CAA CAT TTT CTG CCA TCA CTG AGC CAC

80    L  A  V  V  N  P  V  A  Y  L  Q  Q  Q  Q  L  L  A  S  N  P  L
      CTA GCA GTG GTG AAC CCT GTC GCC TAC TTG CAA CAG CAG CTG CTT GCA TCC AAC CCA CTT

100   A  L  A  N  V  A  T  Y  Q  Q  Q  Q  Q  Q  L  Q  Q  F  M  P  A
      GCT CTG GCG AAC GTA GCT ACA TAC CAG CAA CAA CAA CAG CTG CAA CAG TTT ATG CCA GCG

120   L  S  Q  L  A  M  V  A  P  A  V  Y  L  Q  L  L  S  S  S  P
      CTC AGT CAA CTA GCC ATG GTG AAC CCT GCC GTC TAC CTA CAA CTG CTT TCA TCT AGC CCG

140   L  A  V  G  N  A  P  T  Y  L  Q  Q  Q  L  L  Q  Q  I  V  P
      CTC GCT GTG GGC AAT GCA CCT ACG TAC CTA CAA CAA CAG TTG CTG CAA CAG ATT GTA CCA

160   A  L  T  H  Q  L  A  M  A  N  P  A  T  Y  L  Q  Q  L  L  P
      GCT CTA ACT CAT CAG CTA GCT ATG GCA AAC CCT GCT ACC TAC TTA CAA CAG TTG CTT CCA

180   F  N  Q  L  A  V  S  N  S  A  A  Y  L  Q  Q  R  Q  Q  L  L
      TTC AAC CAA TTG GCT GTG TCG AAC TCT GCT GCG TAC CTA CAA CAG CGA CAA CAA TTA CTT

200   N  P  L  A  V  A  N  P  L  V  A  T  F  L  Q  Q  Q  Q  L  L
      AAT CCA TTG GCA GTG GCT AAC CCA TTG GTC GCT ACC TTC CTG CAG CAG CAA CAA TTG CTG

220   P  Y  N  Q  F  S  L  M  N  P  A  L  Q  Q  P  I  V  G  G  A
      CCA TAC AAC CAG TTC TCT TTG ATG AAC CCT GCC TTG CAG CAA CCC ATC GTT GGA GGT GCC

240   I  F  *
      ATC TTT TAG
```

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 97/11723

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/415 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 885 357 A (LARKINS BRIAN ET AL) 5 December 1989	1,2,4, 7-11
Y	see the whole document	1-12,14, 17

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Y	see the whole document	1-12,14, 17

X	EP 0 319 353 A (PLANT GENETIC SYSTEMS NV) 7 June 1989	1,2,8-11
Y	PAGE 7, LINE 30-45; PAGE 8, LINE 64; PAGE 9, LINE 50-65	1,2,7-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1997	29/12/1997

Name and mailing address of the ISA	Authorized officer
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Inter onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 91 13993 A (UPJOHN CO) 19 September 1991 see example 5 ---	1-17
A	WO 92 14822 A (DU PONT) 3 September 1992 see the whole document ---	1-17
A	LIU, C-N., ET AL . : "MOLECULAR CHARACTERIZATION OF TWO TYPES OF 22 KILODALTON ALPHA-ZEIN GENES IN A GENE CLUSTER IN MAIZE" MOLECULAR AND GENERAL GENETICS, vol. 234, 1992, pages 244-253, XP002048189 see figure 2 ---	1-17
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